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<p>(21) International Application Number: <b>PCT/US97/12982</b></p> <p>(22) International Filing Date: <b>24 July 1997 (24.07.97)</b></p> <p>(30) Priority Data: <b>60/022,658</b>                   <b>26 July 1996 (26.07.96)</b>                   <b>US</b></p> <p>(71) Applicants: THE STATE OF OREGON acting by and through THE STATE BOARD OF HIGHER EDUCATION on behalf of THE UNIVERSITY OF OREGON [US/US]; 311 Hendricks Hall, Eugene, OR 97403-1238 (US). IKONOS CORPORATION [US/US]; Second floor, 320 S.W. 6th Avenue, Portland, OR 97204 (US).</p> <p>(72) Inventors: MINGDI, Yan; 14088 S.E. Summers Court, Clackamas, OR 97015 (US). KAPAPETROV, Goran; 3435 N.W. Orchard, Corvallis, OR 97330 (US). SEVRAIN, Christophe, J.-P.; 21418 N.W. 11th Avenue, Ridgefield, WA 98642 (US). KEANA, John, F.W.; 3854 Onyx Street, Eugene, OR 97405 (US). WYBOURNE, Martin, N.; 1676 Arendale Lane, Eugene, OR 97405 (US).</p> <p>(74) Agent: POLLEY, Richard, J.; Klarquist, Sparkman, Campbell, Leigh &amp; Whinston, LLP, Suite 1600, One World Trade Center, 121 S.W. Salmon Street, Portland, OR 97204 (US).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p><b>Published</b> <i>With international search report.</i></p>	
<p>(54) Title: <b>SENSOR FOR DETECTING HEPARIN AND OTHER ANALYTES</b></p> <p>(57) Abstract</p> <p>A sensor and method of use for detecting heparin in which a flow cell (12) has immobilized therein analyte sensing materials (14), e.g., in linear arrays (16). Samples flow through the cell (12) via an inlet line (18) and exit via an outlet line (22). Flushing solution may be provided by an auxiliary line (20). Binding of analyte to the sensing material (14) is detected, e.g., by surface plasmon resonance. The sensing materials are immobilized by treating the immobilization surface with perfluorophenyl azide compounds.</p>			

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## SENSOR FOR DETECTING HEPARIN AND OTHER ANALYTES

### FIELD OF THE INVENTION

This invention concerns chemical sensors and biosensors, and is particularly directed to sensors for detecting heparin alone, and for detecting  
5 heparin in combination with other analytes.

### GENERAL DISCUSSION OF THE BACKGROUND

A major goal in sensor technology is to develop sensors that are smaller, and yet have greater sensitivity. Also, increasingly stricter specificity and selectivity demands are being imposed, particularly for physiological monitoring,  
10 diagnostic monitoring, bioprocessing, agriculture, pharmaceuticals and therapeutic monitoring.

A "sensor" is a type of transducer; i.e., a device that responds to an external stimulus or input signal by producing a measurable response having a magnitude bearing a relationship to the magnitude of the external stimulus or  
15 input signal.

A "chemical sensor" is a sensor in which a chemical reaction or molecular change in or on the sensor is an important aspect of the production of a measurable response by the sensor.

A "biosensor" is a sensor that incorporates a biological or biomolecular  
20 component as a key functional element in the production of a measurable response by the sensor.

Biosensors have been the subjects of great attention. See, e.g., Vadgama et al., "Biosensors: Recent Trends," Analyst 117:1657-1670 (1992). However, bridging the gap between knowledge of a particular reaction involving  
25 biomolecules and the exploitation of the reaction in a biosensor has often proved difficult. For example, in biosensors the biological component is usually in the form of a biolayer that is frequently metastable. Thus, many contemporary biosensors are subject to obfuscating environmental influences. Secondly, biolayers usually need to directly contact the analyte which is frequently present  
30 in a complex mixture comprising a large number of other compounds that can

interfere with the response of the biolayer to a target analyte or that can be interfacially active and/or possibly detrimental to the biolayer.

An important problem often encountered in making sensors, particularly biosensors, is how to immobilize molecules of the sensing compound (i.e., the "sensing molecules") at a particular location on an appropriate substrate surface. Such immobilization cannot substantially adversely affect the ability of the sensing molecules to respond to significant changes in the measured parameter when the sensing molecules are exposed or otherwise contacted with molecules of an analyte. Immobilizing sensor molecules on a sensor requires that the sensing molecules retain their reactive specificity toward the corresponding analyte when the sensing molecules are attached to the situs. Otherwise, the function and/or specificity of the sensor may be compromised.

One way to immobilize sensing molecules to a situs is to chemically bond them to the situs. However, particularly with biomolecules, immobilizing sensing molecules on a sensor substrate by conventional bonding techniques can cause the sensing molecules to undergo conformation changes or undergo any of several other changes that can reduce or destroy the capacity of the sensing molecules to respond to the analyte.

Another problem often encountered is that while many substrates have properties that render them desirable for use as substrates, it is often difficult or impossible by contemporary methods to bond sensing molecules to them. This is particularly true when considering that the chemistry used to bond sensing molecules to substrates cannot damage the substrate, the sensing molecules, or both. Attaching sensing molecules to a substrate can be thought of as a form of chemical modification of, or "functionalization" of, a substrate. Sensors useful for continuously monitoring heparin levels currently are needed. Heparin, a naturally-occurring carbohydrate that acts as an anticoagulant, is a polymer of O- and N-linked sulfated glucosamines and hexuronic acids that are linked by glycoside bonds. "Principles of Pharmacology: Basic Concepts and Clinical Application," edited by Paul Munson et al., (Chapman and Hall, 1995). Commercial heparin preparations have mean molecular weights ranging from

about 15,000 to about 18,000. Although most of the activity appears to be associated with lower molecular weights, it is believed that at least 16 to 20 monosaccharides are required for full expression of heparin activity. Id.

Heparin has an immediate prolongation of plasma clotting time, and hence 5 is the anticoagulant of choice for thromboembolic disease. Id. However, there are complications associated with the use of heparin, the most serious of which is unwanted bleeding. This requires constant monitoring of the patient, and continuous blood sampling, in order to ward off any serious complications arising from the administration of heparin to patients.

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#### SUMMARY OF THE INVENTION

The need for a heparin sensor, and a sensor that can monitor heparin in combination with other analytes, is met by the present invention. The invention involves methods for covalently functionalizing materials, including the surfaces thereof, to include sensing materials useful for sensing heparin and other 15 analytes. The invention also concerns detecting analytes once they interact with sensing molecules. Examples, without limitation, of sensing materials for sensing heparin include protamine, heparinase, and other nitrogen containing materials.

A number of substrates can be functionalized to include such sensing 20 molecules. Substrates that can be functionalized according to the method of the present invention include, but are not limited to: a wide variety of polymeric materials, such as polyoctyl-3-thiophene, polystyrene, polypropylene, polyethylene, polyphenol, polyimide, PMMA, and C<sub>60</sub>; various allotrophic forms of elemental carbon (e.g., graphite, "carbon electrodes," diamond and diamond 25 films, and fullerenes such as C<sub>60</sub> and C<sub>70</sub>); siliceous materials; semiconductor materials, such as silicon, gallium arsenide, cadmium sulfate, and other semiconducting materials (doped or not doped); and metals, such as gold, aluminum, platinum, silver, copper iron, and alloys containing these metals, such as steel.

30 Substrate surfaces are functionalized by exposing the surface to a nitrenogenic functionalizing reagent in the presence of a reaction-energy source

such as photons, electrons and/or heat. In the presence of the reaction-energy source, the functionalizing reagent forms a nitrene intermediate that covalently reacts with -CH, -NH, -OH, -C=C-, -C-C- and other groups on the substrate surface so as to cause "nitrene addition" or "nitrene insertion" of the  
5 functionalizing reagent to the substrate surface. In order to form nitrene intermediates, the functionalizing reagent must terminate with an azide group or analogous chemical group capable of forming a reactive nitrene when exposed to a reaction-energy source.

The substrate and/or substrate surface is functionalized via either a single-  
10 stage or a multi-stage process. In a multi-stage process, each stage typically involves different functionalizing reagents. In both single- and multi-stage processes, at least one stage involves a nitrenogenic functionalizing reagent.

A class of preferred functionalizing reagents for single- and multi-stage processes according to the present invention consists of N-hydroxysuccinimide  
15 active ester-functionalized perfluorophenyl azides (NHS-PFPAs). The NHS active ester groups become covalently attached to the substrate via generation during the reaction of highly reactive nitrene intermediates derived from the PFPAs portion of the reagent molecules. (The reactive nitrene portion of the intermediates are preferably constrained structurally such that the nitrene portion  
20 cannot react intramolecularly with the NHS active ester portion.) Thus, the substrate, either the surface thereof or throughout the substrate cross-section, becomes "modified" (i.e., "functionalized"). Afterward, the active esters can participate in further reactions with a variety of nucleophilic reagents, such as reagents containing primary amines or hydroxyls (such as biomolecules) by way  
25 of amide or ester formation, respectively.

According to another aspect of the present invention, a nitrene-forming functionalizing reagent can be applied, such as in the form of a film, to the substrate surface. Alternatively, a mixture comprising molecules of a nitrene-forming functionalizing reagent and polymer molecules can be applied, such as in  
30 the form of a film, to the surface of a substrate. Then, the coating, film or coated surface is exposed to a reaction-energy source (such as photons or a beam

of particles such as an electron beam) in a spatially selective way to functionalize certain regions of the surface and not others, thereby creating a functionalized pattern on the surface. Such patterns can have dimensions measured in micrometers and smaller, due to the highly resolved manner in which the coated 5 surface can be exposed to the reaction-energy source. Thus, the present invention has wide applicability in microelectronics and in the construction of novel micron-scale biosensors.

A particularly suitable PHPA is an N-hydroxysuccinimide active ester-functionalized perfluorophenyl azide (NHS-PFPAs). The NHS-PFPA provides an 10 activated ester (i.e., an ester that is more reactive to nucleophilic attack than an alkyl ester or a carboxylic acid) that can be reacted with a nucleophilic sensing molecule e to couple the nucleophile to the substrate. Virtually any nucleophile could be reacted in this manner; however, by way of example only and without limitation, the nucleophile may be selected from the group consisting of peptides, 15 nucleotides, cells and antibodies.

A sensor that includes sensing molecules for detecting heparin, and heparin in combination with other analytes of interest, can be made by coupling a functionalized substrate having sensing molecules for heparin, such as, without limitation, protamine, and for the other selected analytes with a detection system 20 for detecting when the sensing molecules interact with the analytes. A working embodiment of a sensor included protamine as sensing molecules for sensing heparin, and may also have included glucose oxidase for sensing glucose. One embodiment of the invention uses a substrate, such as a one having a metal surface, particularly a silver or gold surface, wherein the surface of the metal has 25 been functionalized to include selected sensing molecules. This substrate was coupled to a surface plasmon resonance detector for detecting when the protamine sensing molecules interacted with samples containing analytes.

One embodiment of a sensor according to the present invention comprises an analyte detector comprising sensing materials immobilized thereon for 30 ionically or covalently binding heparin. The sensing materials typically, but not necessarily, are selected from the group consisting of protamine, heparinase,

polylysine, poplybrene, quaternary ammonium salts, and mixtures thereof.

Detector means, such as surface plasmon resonance means, are coupled to the analyte detector for detecting heparin after it binds to the sensing materials. The sensor can be configured to monitor or detect analytes other than heparin.

- 5 Moreover, the sensor can include means for reversing the interaction of heparin with the sensing molecules.

The sensors can be small and therefore configured as a hand-held unit or as implantable devices. Alternatively, the sensor may further comprise at least one catheter fluidly coupled to the sensor and to a patient for flowing blood from the 10 patient to the analyte detector. With such devices, an analyte pump can be fluidly coupled to the patient for administering analyte to a patient when analyte levels detected by the sensor are lower than a predetermined threshold.

The present invention also provides a method for detecting heparin and other analytes. One embodiment of the method comprises first providing a

15 substrate capable of undergoing an insertion reaction with a nitrene. The substrate is then coated with an activated perfluorophenyl azide. The substrate and perfluorophenyl azide are then exposed to a reaction energy source, thereby forming a functionalized substrate. The functionalized substrate is reacted with heparin sensing molecules, thereby forming a heparin sensitive substrate. A

20 detector is coupled to the heparin sensitive substrate to form a sensor, which is then exposed to samples containing heparin so that the sensing molecules interact with the heparin. The sensing molecule-heparin interactions are then detected using a detector, such as an SPR detector.

#### BRIEF DESCRIPTION OF THE DRAWINGS

25 FIG. 1A is an image obtained with an atomic-force microscope of a freshly cleaved graphite surface functionalized first with NHS-PFPA, then with horseradish peroxidase, as described in Example 1.

FIG. 1B is an atomic-force microscope image of an experimental control wherein a freshly cleaved graphite surface was treated with horseradish 30 peroxidase but not with NHS-PFPA, as described in Example 1.

FIG. 2A is a photomicrograph obtained using a fluorescence microscope of circular patterns produced on a film of poly(3-octylthiophene) and 7 wt-% of NHS-PFPA by exposing the film to electron-beam lithography conditions and subsequently treating the film with amino-fluorescein, wherein the microscope  
5 was fitted with a rhodamine filter set.

FIG. 2B is a photomicrograph obtained using a fluorescence microscope of circular patterns produced on a film of poly(3-octylthiophene) and 7 wt-% of NHS-PFPA by exposing the film to electron-beam lithography conditions and subsequently treating the film with amino-fluorescein, wherein the microscope  
10 was fitted with a fluorescein filter set.

FIG. 2C is a photomicrograph obtained using a fluorescence microscope of circular patterns produced on a film of poly(3-octylthiophene) by exposing the film to electron-beam lithography conditions and subsequently treating the film with amino-fluorescein, wherein the microscope was fitted with a rhodamine  
15 filter set.

FIG. 2D is a photomicrograph obtained using a fluorescence microscope of circular patterns produced on a film of poly(3-octylthiophene) by exposing the film to electron-beam lithography conditions and subsequently treating the film with amino-fluorescein, wherein the microscope was fitted with a fluorescein  
20 filter set.

FIG. 3 is a schematic plan view of a flow-through cell useful for analyzing analytes.

FIG. 4 is a schematic cross-sectional side view of a sensor substrate and detection system.

25 FIG. 5 is schematic illustrating one embodiment of an SPR detection system.

FIG. 6 is a graph illustrating a sample reflectance curve that was obtained using the detection system of FIG. 5.

30 FIG. 7 is a graph illustrating the time dependence of the SPR minima as the sensor is subjected to different substances.

FIG. 8 is a graph illustrating the SPR position dependence on the heparin exposure time.

### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

#### I. INTRODUCTION

5 This invention concerns methods and sensors for detecting analytes, particularly heparin-sensors alone or sensors that are capable of detecting plural analytes, such as heparin and glucose. The sensors can be irreversible, and hence disposable, or reversible with respect to the binding or other interaction between the sensor and the analytes being detected. The basic approach is to  
10 immobilize heparin sensing material or materials, or heparin sensing material(s) in combination with sensing material(s) for other analytes, on a substrate. Typical substrates include, without limitation, glass, glass coated with a metal or metals, chips made from semiconducting materials, polymeric materials, or coated polymeric materials. The substrate is then coupled to a detection system  
15 for detecting the analytes of interest.

#### A. Heparin

Heparin is a charged, sulphated glycosaminoglycan found in liver, lung and other tissues. Heparin has several pharmacological properties [Nader, H.B. & Dietrich, C.P. Heparin, Chapter 5, Edward Arnold, London (1989)]. The  
20 property of heparin which has attracted most attention and resulted in its widespread use is its ability to prolong blood clotting times, presumably by preventing the formation of fibrin. The anticoagulant activity of heparin is used in vascular surgery and in treatment of postoperative thrombosis and embolism. A heparin treatment must result in heparin levels in the blood that are sufficient  
25 to control thrombosis and yet avoid undue risk of bleeding. The antithrombotic effect and the risk of bleeding varies, however, not only with the dose, but also with the individual. This variation in response to heparin calls for an individualization of the heparin dose regimen and a careful clinical monitoring by laboratory test. [Abildgaard, U, Heparin, Chapter 23, Edward Arnold, London  
30 (1989)].

No consensus of opinion exists concerning which is the most useful test for monitoring heparin treatment. It was observed that when heparin was injected and subsequently separated from the blood, the separated heparin had higher anticoagulant activity per mg than the same heparin before injection (Levym  
5 S.W. et al., Thrombin Research (1978), 13:429-441 and Bjornsson, T.D. et al., European Journal of Clinical Pharmacology, (1982), 21:491). This indicates that there are important differences between the measurement of absolute concentrations of heparin and the amount of heparin present as determined by an  
10 *in vitro* coagulation test based on a calibration curve. Therefore, it is preferable to have a method for determining the total absolute amount of heparin in plasma (Jaques, L.B. et. at, Journal of Laboratory Clinical Medicine, (1990), 115, 422-432).

#### B. Heparin Sensing Molecules

Any material that is capable of interacting with heparin, either ionically or  
15 covalently, and either reversibly and irreversibly, are potential candidates for use as heparin sensing materials. Examples, without limitation, of heparin sensing molecules include protamine, heparinase, and polycationic macromolecules known to bind heparin, for example, polylysine and poplybrene. Long-chain quaternary ammonium salts, such as tridodecylmethylammonium chloride, also  
20 bind heparin electrostatically.

These sensing molecules can be immobilized on a substrate to serve as the affinity ligands for heparin. The advantage of using protamine or the polycationic macromolecules is that the electrostatic interaction between these reagents and heparin can be reversed, thereby allowing for the formation of  
25 reversible sensors. The electrostatic interaction can be reversed by soaking or flushing the substrate or sensor in concentrated saline solution to back-extract the heparin and regenerate the immobilized sensing reagents.

Protamine currently is a preferred example of a heparin sensing material that forms reversible electrostatic interactions with heparin. Protamine is a very  
30 basic protein with a molecular weight of about 4,000-4,500, typically containing 20 arginines out of 30 amino acids. Protamine interacts with heparin through a

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1:1 pairing of anionic heparin sites with cationic protamine sites. The binding affinity is  $7 \times 10^7 \text{ M}^{-1}$ . Yun J.H. et al., Journal of Electroanalysis, 5:719 (1993). Protamine is used clinically to reverse the anticoagulant activity of heparin. Casu, B., Heparin and Related Polysaccharides, Structure and

5 Activities, The New York Academy of Sciences, New York, (1989).

Non-reversible sensors can be made by immobilizing molecules which interact with heparin irreversibly. One example is heparinase. Heparinase is an eliminase which cleaves certain  $\alpha$ -glycosidic linkages in heparin. Heparinase can be immobilized on a substrate using an NHS-PFPA ester via the reaction of NHS esters with the amino groups present in heparinase.

### C. Detection Methods

There are several transduction methods that can be used to detect association of the analyte with the sensor including, without limitation, optical methods, spectrophotometric methods, methods involving measuring changes in resistivity or capacitance and surface plasmon resonance. A currently preferred method for such detection is SPR, which is described in detail below. SPR is sensitive to the thickness and index of refraction of material at the interface between a thin metal film (such as gold or silver) and a bulk medium (air). By immobilizing protamine as the affinity ligand, the interaction of protamine and heparin can be detected using SPR because the interactions induce changes in both index of refraction and the thickness of the film.

The following paragraphs provide definitions of certain terms used in this application and describe general methods for functionalizing substrates with sensing molecules. Means for detecting analytes once they are associated with sensing molecules immobilized on substrate surfaces also are described.

## II. DEFINITIONS

The following terms are used herein:

A "substrate" is a non-fluid material providing a surface that can be functionalized according to the present invention. A substrate can comprise polymer molecules (e.g., thermoplastic polymer molecules), a thermoset

30

molecular network (e.g., cross-linked polymer molecules), or other atomic or molecular associations such as found in certain glasses and crystals.

A "surface molecule" is a substrate molecule having at least a portion thereof present on the substrate surface.

5 A "polymeric material or substrate" is a substrate comprising polymer molecules or a network of polymer molecules.

A "polymer molecule" is a relatively large molecule formed by covalently linking smaller molecules termed "monomers." The monomers present in a polymer molecule can be the same or different. Polymer molecules can be  
10 natural, such as (but not limited to) cellulose, starch, proteins, and nucleic acids; or synthetic such as (but not limited to) nylon and polyethylene. In a polymeric material, polymer molecules can be associated with each other in any of several ways, including non-covalently (as a thermoplastic) or a covalently cross-linked network (as a thermoset).

15 A "functionalized substrate" is a substrate to which one or more functional groups are covalently bonded according to the methods of the present invention.

A "functional group" is a group of one or more atoms bonded together in an organized way so as to have a desired chemical property. Certain functional groups can, when covalently bonded to a substrate surface, participate in one or  
20 more additional bonding reactions with either a similar functional group or a different type of functional group. Such bonding reactions can result in: (a) attachment to the functional groups of any of a variety of additional functional groups; or (b) coupling together (cross-linking) of the functionalized substrate molecules. Many other functional groups attachable to polymer molecules  
25 according to the present invention can confer altered chemical properties to the polymer molecules such as, but not limited to, making them labeled or tagged with a fluorescent, radioactive, immunologic, diagnostic or therapeutic markers.

The term "functionalized polymer" can concern either a functionalized polymeric substrate or a functionalized polymer molecule. Functionalized  
30 polymer molecules comprise one or more functional groups covalently bonded to the polymer molecules according to the present invention.

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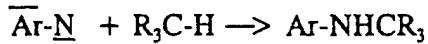
A "functionalizing reagent" according to the present invention is a reagent adapted for functionalizing a substrate. Molecules of functionalizing agents have at least one nitrenogenic group (as a first functional group) coupled to a second functional group. The nitrenogenic group preferably is constrained by the 5 molecular structure of the functionalizing-reagent between the nitrenogenic group and the functional group. The nitrenogenic groups are capable under reaction conditions of functionalizing a substrate surface.

A "nitrenogenic group" on a functionalizing reagent is a chemical moiety that, when exposed to a reaction-energy source, becomes a nitrene group.

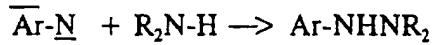
10 The phrase "addition reaction" when used in the context of reactions of the nitrene group of the functionalizing reagents with surface molecules, generally refers to any of the various addition and insertion reactions that nitrenes can undergo with molecules on the substrate surface according to the present invention.

15 A "nitrene group" (also generally termed "nitrene" or "nitrene intermediate") is a particular form of nitrogen group that can be depicted as a singlet by the structure:  $\overline{\text{R-N}}$ , and as a triplet by the structure:  $\overline{\text{R-N}}$ . Nitrenes are regarded by persons skilled in the art as the nitrogen analogs of carbenes. Like carbenes, nitrenes are generally regarded as intermediates that are highly reactive 20 and generally cannot be isolated under ordinary conditions. However, certain chemical reactions, such as reactions according to the present invention, would not otherwise be explainable by known reaction mechanisms without the presumed existence of nitrenes. Important nitrene reactions can be summarized by the following:

25 (a) Nitrenes, including aryl nitrenes, can undergo addition reactions at -CH sites and at -NH sites; e.g.:



30

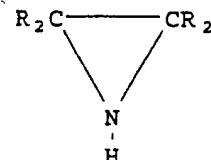


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(b) Nitrenes can also undergo addition at -C-C- and -C=C- bonds; e.g.:



5



10

According to the present invention, a functionalizing reaction occurs when a functionalizing reagent comprising a nitrenogenic group is exposed to a reaction-energy source, which converts the nitrenogenic group to a nitrene intermediate. The functionalizing reaction proceeds by reaction of the nitrene intermediate with the substrate surface.

15 A "reaction-energy source" is an energy source that drives a functionalizing reaction according to the present invention by, in particular, converting nitrenogenic groups on functionalizing reagent molecules to nitrenes which react with the substrate surface. Suitable reaction-energy sources include (but are not limited to): photons (such as ultraviolet (UV) light, deep-UV light, laser light, X-rays, and heat in the form of infrared radiation or conductive heating), energized electrons (such as an electron beam), and energized ions (such as an ion beam). These reaction-energy sources are conventionally used for such tasks as lithography, scanning microscopy, and, in the case of UV and visible photons, 20 effecting photochemical reactions and excitation of fluorescent molecules.

25 A "functionalizing reaction" is a reaction in which a substrate surface is functionalized according to the present invention. A functionalizing reaction can consist of one or more stages. At least one stage involves the reaction in the presence of a reaction-energy source of the substrate surface with molecules of a functionalizing reagent comprising nitrenogenic groups.

### **III. GENERAL FUNCTIONALIZATION METHODOLOGY**

According to the present invention, a substrate is functionalized by a chemistry whereby functional groups on functionalizing reagent molecules

become covalently bonded to the substrate or substrate surface. Such covalent bonding is achieved by conversion of nitrenogenic groups on the functionalizing reagent molecules (the functionalizing reagent molecules also each comprising a desired functional group as set forth below) to a nitrene intermediate highly

- 5 reactive with the substrate surface by exposing the functionalizing reagent molecules to a reaction-energy source.

The functionalizing reagent is preferably selected from a group consisting generally of: aryl azides, alkyl azides, alkenyl azides, alkynyl azides, acyl azides, and azidoacetyl derivatives, all capable of carrying a variety of substituents.

- 10 Halogen atoms are present to the maximum extent possible in the positions on the functionalizing reagent molecule adjacent the azide group. Best results are achieved when fluorine and/or chlorine atoms are the halogen atoms.

Each of the foregoing azides may also contain within the same molecule any of the following functional groups, constrained structurally from reacting

- 15 with the nitrene moiety after the nitrene moiety is generated:

(a) carboxyl groups and various derivatives thereof such as (but not necessarily limited to): N-hydroxysuccinimide esters; N-hydroxybenztriazole esters; acid halides corresponding to the carboxyl group; acyl imidazoles; thioesters; p-nitrophenyl esters; alkyl, alkenyl, alkynyl and aromatic esters,

- 20 including esters of biologically active (and optically active) alcohols such as cholesterol and glucose; various amide derivatives such as amides derived from ammonia, primary, and secondary amines and including biologically active (and optically active) amines such as epinephrine, dopa, enzymes, antibodies, and fluorescent molecules;

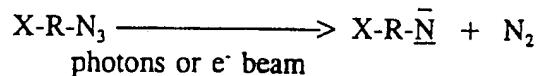
- 25 (b) alcohol groups, either free or esterified to a suitable carboxylic acid which could be, for example, a fatty acid, a steroid acid, or a drug such as naprosin or aspirin;

(c) haloalkyl groups wherein the halide can be later displaced with a nucleophilic group such as a carboxylate anion, thiol anion, carbanion, or

- 30 alkoxide ion, thereby resulting in the covalent attachment of a new group at the site of the halogen atom;

- (d) maleimido groups or other dienophilic groups such that the group may serve as a dienophile in a Diels-Alder cycloaddition reaction with a 1,3-diene-containing molecule such as, for example, an ergosterol;
- (e) aldehyde or ketone groups such that subsequent derivatization is possible via formation of well-known carbonyl derivatives such as hydrazones, semicarbazones, or oximes, or via such mechanisms as Grignard addition or alkylolithium addition; and
- (f) sulfonyl halide groups for subsequent reactions with amines, for example, to form sulfonamides.

10 A general reaction by which a functionalizing reagent is converted to a nitrene intermediate is:



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where X is the functional group and R is an aromatic ring, heteroaromatic ring, or other carbon-containing fragment.

20 A reaction-energy source comprising UV light can be supplied to the reaction by, for example, one of the following representative procedures:

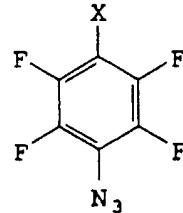
- (a) A sample comprising functionalizing reagent molecules and a substrate is placed in a well of a Rayonet Photochemical Reactor fitted with lamps which emit light of a wavelength suitable for converting the nitrenogenic group into a nitrene, such as 350-nm, 300-nm, or 254-nm lamps. The substrates and reagent molecules are irradiated at ambient temperature for several minutes under air. The duration of the irradiation can be adjusted to change the exposure dose.
- (b) The sample is irradiated through a high-resolution photomask, for example, by (but not limited to) projection UV lithography. In this manner, preselected patterns of functional groups can be immobilized on a substrate.
- 30 (c) Photolysis is carried out in a KSM Karl Suss deep-UV contact aligner using a contact high-resolution photomask. It will be readily appreciated by persons skilled in the art that such procedures can also be generally used to provide the functionalizing reaction with photons of wavelengths other than UV.

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A reaction-energy source comprising electrons can be supplied to the reaction by the following representative procedure: A sample is irradiated under vacuum by an electron or particle beam with an energy selected within the range 1-40 kV. (A representative electron-beam source is a JEOL 840A electron microscope modified for electron-beam lithography.) The beam may be stepped across the surface of the treated substrate to expose certain areas and not others. A dwell time at each step can be adjusted to change the exposure dose.

Particularly effective functionalizing reagents are selected from the group consisting of perhalophenyl azides (PHPAs), particularly perfluorophenyl azides (PFPA) derived from 4-azido-2,3,5,6-tetrafluorobenzoic acid in which the carbonyl group is further activated through reactive ester, amide, acid halide, or mixed anhydride formation. For example, and not intended to be limiting, representative functionalized perfluorophenyl azides have the general structure:

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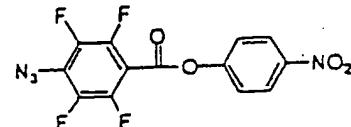
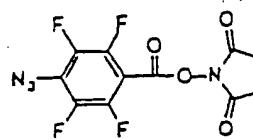


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wherein X can be any of the following: CN, CONH<sub>2</sub>, CHO, CO<sub>2</sub>Me, COMe, NO<sub>2</sub>, CO<sub>2</sub>H, COCl, CO-Imidazole, CONHS, CH<sub>2</sub>OH, CH<sub>2</sub>NH<sub>2</sub>, COCH<sub>2</sub>Br, N-maleimido, NH-biotinyl, CONH-R (where R is a polypeptide moiety), CONH-X-S-S-Y-NH-biotinyl (where X and Y are spacer atoms and the S-S bond is reductively cleavable at a later stage), and CONHS-SO<sub>3</sub>Na.

Representative activated PFPA's include (but are not limited to) the N-hydroxysuccinimide (NHS) ester A (also designated "NHS-PFPA"), the p-nitrophenyl ester B, the 1-hydroxybenzotriazole ester C, the acyl imidazole D, the acid chloride E, the mixed anhydride F and the 2,2,2-trichloroethyl ester G:

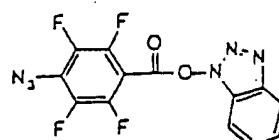
5



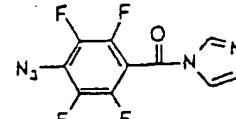
A

B

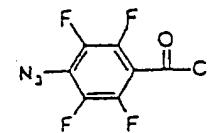
10



C

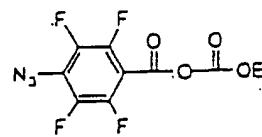


D

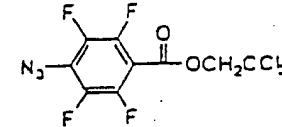


E

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F



G

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In addition to the foregoing candidate functionalizing reagents, it is possible to utilize other PPAs having "spacers" situated between the reactive functional group and the PFPAs moiety. Other candidate aryl azides useful as functionalizing reagents are similar to the above examples except that another aryl moiety replaces the PFPAs.

Candidate substrates that can be functionalized according to the present invention include, but are not limited to: polymeric substrates, graphite, metals,

25 and siliceous materials; as well as silicon, gallium arsenide, and other semiconducting materials.

30 In the case of siliceous substrates (e.g., glass, silica, mica, quartz) it is believed that the functionalizing reagents, when converted to corresponding nitrenes, react with Si-O-H groups, Si-OH groups, or Si-OSi groups on the substrate surface.

In the case of graphite and other allotrophic forms of elemental carbon, it is believed that the functionalizing reagents, when converted to the corresponding nitrenes, react with carbon rings on the substrate surface.

Polymeric substrates that can be functionalized according to the present invention include virtually any polymeric material comprising polymer molecules possessing -CH groups, and/or -NH groups, and/or -OH groups and/or -C=C-sites. Such polymeric substrates include, but are not limited to:

- (a) saturated polyolefins as exemplified by polyethylene, polyvinyl chloride, polytetra-fluoroethylene, polypropylene, polybutenes, and copolymers thereof;
- (b) acrylic resins such as polymers and copolymers of acrylic acid, methacrylic acid [eg., poly(methyl-methacrylate), poly(hexylmethacrylate)], and acrylonitrile;
- (c) polystyrene and its analogues such as poly(p-chlorostyrene) and poly(p-hydroxystyrene);
- (d) unsaturated polyolefins such as poly(isoprene) and poly(butadiene);
- (e) polyimides such as polyimide(benzophenone tetracarboxylic dianhydride/tetraethylmethylenedianiline);
- (f) polyesters such as poly(trimethylene adipate) and poly(hexymethylene sebacate);
- (g) conjugated and conducting polymers such as poly(3-alkylthiophene), poly(3-alkylpyrrole), and polyaniline;
- (h) inorganic polymers such as poly(aryloxyphosphazene), poly[bis(trifluoro-ethoxy)phosphazene], polysilanes, and polycarbosilanes, siloxane polymers, and other silicon-containing polymers;
- (i) organic metals (i.e., organic polymers with metallic properties) such as polycroconaines and polysquaraines, as described in Chemical and Engineering News (August 31, 1992), p.8.
- (j) organometallic polymers such as palladium poly-yne and ferrocene-containing polyamides; and
- (k) polysaccharides such as cellulose fibers, chitin, and starch.

Functionalizing substrates according to the method of the present invention requires that molecules of the functionalizing reagent and the substrate be brought into "reactive proximity"; i.e., brought together sufficiently closely so as to undergo a functionalizing reaction when exposed to the reaction-energy source.

- 5 One way materials, such as polymers, can be functionalized is to prepare a solution comprising the material and the functionalizing reagent. Another way is to prepare a suspension or mixture comprising the functionalizing reagent and substrate particles or substrate agglomerations. Yet another way is to apply the functionalizing reagent (such as a solution of the functionalizing reagent in a
- 10 solvent capable of absorbing into the substrate) to a surface of the substrate, then allowing the functionalizing reagent to absorb into the substrate.

Functionalization of a substrate can occur in one or more stages, depending upon various factors such as the particular material to be functionalized; the form of the material (i.e., solution, particulate suspension, non-fluid mass); the

- 15 functional group(s) to be attached to the polymer molecules; the necessity to protect the functional groups from undesired reactions during reaction of the functionalizing reagent with the polymer molecules; and on other matters.

- For example, in a one-stage functionalization, substrate molecules and molecules of a functionalizing reagent each having a nitrenogenic group and a
- 20 desired functional group are brought into reactive proximity. Upon exposure to a reaction-energy source, the nitrenogenic groups are converted to nitrenes which react with -CH, -NH, -OH, -C=C-, C-C, and other groups on the substrate molecules reactive with nitrenes, thereby covalently bonding the functional groups to the substrate molecules. The functional groups typically do not require
- 25 additional chemistry performed on them to confer the desired useful property to the resulting functionalized substrate.

- In a two-stage functionalization protocol, each stage involves a different functionalizing reagent. The first stage involves a first functionalizing reagent, such as, without limitation, an NHS-PFPA. The first functionalizing reagent is
- 30 converted during the course of the first-stage reaction to a nitrene intermediate. During the first stage using, for example, a polymeric substrate, the NHS active-

ester groups on the NHS-PFPA molecules become covalently attached to surface polymer molecules.

As another example of a two-stage functionalization reaction, the first stage may involve a first functionalizing reagent such as an NHS-PFPA compound.

- 5 Upon exposure to a reaction-energy source, the azide group of the PFPA portion is converted to a nitrene intermediate that reacts with polymer molecules. Thus, the NHS active-ester groups on the NHS-PFPA molecules become covalently attached to the polymer molecules. Thus, this first-stage reaction requires generation of a highly reactive nitrene intermediate derived from the NHS-PFPA  
10 by exposure of the NHS-PFPA to a reaction-energy source.

As still another example of a two-stage reaction protocol, the first stage can be performed by interspersing molecules of a first functionalizing reagent depthwise into the substrate mass, such as by first forming a fluid solution or suspension comprising the polymer and the first functionalizing reagent; forming

- 15 the fluid into a desired shape; then converting the fluid into a product having a rigid form. The reaction-energy source is then applied to the rigid product to covalently bond the first functionalizing reagent to the polymer molecules.  
Subsequently, during the second stage, the second functionalizing reagent is applied to a surface of the rigid product.

- 20 As can be seen by the preceding examples, the NHS-ester portions of the PFPAs do not participate in this first-stage chemistry. Rather, the NHS-esters, after being transferred to the surface molecules, are utilized in second-stage chemistry, discussed below.

- In the second stage, the NHS esters readily react with molecules of a  
25 second functionalizing reagent. The second functionalizing reagent is selected from a group consisting of molecules possessing primary or secondary nucleophilic species, such as, but not limited to, amines, sulfhydryls, and/or hydroxyls. Reaction of NHS-esters with hydroxyls proceeds via ester formation.

- Since many types of biological molecules possess nucleophilic groups, such  
30 as amine and/or hydroxyl groups, these molecules can serve as functionalizing reagents adapted for reaction in a second-stage functionalization reaction with

NHS-esters covalently bonded to the surface molecules in a first-stage functionalization reaction. Thus, it is possible to attach any of a wide variety of molecules, including macromolecules such as proteins, nucleic acids, carbohydrates, and various other molecules, to substrates and/or surfaces thereof 5 using methods according to the present invention. Thus, the invention provides methods for immobilizing heparin sensing molecules to substrates.

By practicing the methods of the present invention, it also is possible to first prepare nitrenogenic derivatives of molecules (such as biomolecules, drugs, analytes, catalysts [including transition metals], and diagnostic agents) to be 10 attached to the substrate, bring the derivatives into reactive proximity or apply the derivatives to a surface of the substrate, then expose the derivatives or treated surface to a reaction-energy source to cause the nitrenogenic derivatives to covalently bond to the substrate and/or surface molecules via nitrene intermediates. It is necessary for the nitrenogenic moiety to be structurally 15 constrained such that the nitrene cannot readily react with another part of the same molecule. For instance, with NHS-PFPA functionalizing reagents the 4-position of the phenyl ring is the preferred position for the azide group.

#### IV. DETECTING ANALYTES

The present invention relies on first having analytes of interest associate, 20 such as through ionic or covalent bonding, with sensing materials immobilized on portions of a sensor, and thereafter detecting the presence of the analyte in its associated state with the sensing portion. Working embodiments of the invention have used SPR as the detection method.

##### A. SPR

Without limiting the invention to one theory of operation, it is believed that 25 SPR operates in the following manner. On the plasma surface (usually a material with a relatively large imaginary part and small real part of the optical dielectric constant, i.e. metals such as silver or gold) collective resonating oscillations of free electrons can be established. This produces a charge density wave propagating along the plasma surface. This surface plasmon wave can be excited 30 by an electromagnetic wave traveling through the medium coated on the metal.

Light can be used to excite the SPR. The light should be transverse magnetic (TM) polarized due to the specific boundary conditions at the metal - dielectric interface. When the wave vector and the frequency of the incident light coincide with those of the surface plasmon the light resonantly excites the surface

5 plasmon. The dispersion relation of the surface plasmon depends on the dielectric constant of the metal and the dielectric of the material in proximity to the metal, such as a metal substrate and any material on top of the metal. By monitoring the resonance condition of the plasmon resonance the dielectric constant of the overlaying material can be obtained. When the overlaying

10 material binds to or somehow otherwise affiliates with different chemical species its dielectric constant changes, which causes a shift in the resonance.

SPR as applied for the detection of surface bound or associated analytes, and perhaps for determining the concentration of such analyte, uses a chemical transduction layer which modifies the surface of the metal. This may enhance

15 the sensitivity and selectivity of the method when the concentration of the analyte is small. Metal surfaces were functionally modified using the methods described above and exemplified below so that such surfaces could bind to specific analyte molecules, thereby locally increasing the concentration of the molecules of interest. This enhanced changes of the dielectric constant at the metal-dielectric

20 interface, which resulted in larger changes in surface plasmon resonance position.

The resonance condition can be monitored in two ways. First, the light wavelength can be fixed and the reflectivity versus the incident angle measured. Attenuation in the intensity of the reflected light occurs at the angular position when the resonance condition is satisfied. At this angle all the energy of the

25 incident beam is taken by the surface plasmon excitation. Second, it is possible to fix the angular position and scan the light frequency. This will again give attenuation at certain wavelengths when the surface plasmon wave vector satisfies the resonance condition. The first approach is a currently preferred approach. The second approach gives similar results but the resulting detection apparatus is

30 more complicated.

### B. Sensor with Flow-through Cell and SPR Detector

FIGS. 3 and 4 illustrate the sensing portion 10 of a heparin sensor. FIG. 3 illustrates that the sensing portion 10 includes a flow-through cell 12 and a substrate 14 having analyte sensing materials immobilized thereon in the manner described above. Flow-through cell 12 was machined from TEFLON, and sealed with O-rings. The substrate 14 may have solely sensing materials for heparin, or may include plural, different sensing materials for sensing plural analytes. Moreover, substrate 14 may have sensing materials immobilized randomly on the surface of substrate 14, or may have sensing materials immobilized on the surface of substrate 14 in preselected patterns, such as the linear arrays 16 illustrated in FIG. 3.

The heparin sensor can be produced to have a continuous flow-through arrangement. In such a system, there must be means for delivering sample containing heparin, such as blood, to the sensing portion 10. Furthermore, it also may be desirable to include flushing capability to the sensing portion 10 of the sensor so that the flow-through cell 12 can be flushed after a sample containing analytes is flowed through flow-through cell 12. As shown in FIG. 3, the sensor can include plural sample delivery lines 18 fluidly coupled to the flow-through cell 12 for delivering sample containing analyte to the cell 12. FIG. 3 also illustrates that a flush line 20 may be fluidly coupled with the flow-through cell 12 for delivering a flushing liquid, such as water or buffer, to the cell 12. Sample, and flushing liquid, is removed from the flow-through cell 12 through exit lines 22. Sample delivery line(s) 18, flush line(s) 20, and exit line(s) 22 were made from TEFLON in a working embodiment.

In operation, sample containing analyte is flowed through flow-through cell 12 through supply lines 18. This is accomplished by actuating an upstream valve (not illustrated) that feeds supply line 18. Once the sample containing analyte is flowed through the flow-through cell 12, upstream sample valve is closed, and an upstream valve is actuated that feeds flush line 20, thereby flushing flow-through cell 12 with, for example, water or buffer solution.

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In some embodiments, a third supply line can be fluidly coupled to the flow-through cell 12. The third supply line can be used to delivering an inert gas, such as N<sub>2</sub>, to the flow-through cell. The inert gas supply line (not illustrated) generally is used to prevent certain analytes from reacting with air, 5 and also may be used to dry the flow-through cell after it is flushed with water or buffer using flush line 20.

FIG. 4 illustrates a side cross-sectional view of sensing portion 10 as illustrated in FIG. 3. FIG. 4 also shows that the flow-through cell portion 12 is covered by a metal-coated surface-plasmon unit 24. A working embodiment of 10 unit 24 comprised a multilayer system made from BK-7 glass as a substrate, which had a thin layer of silver metal and organic film deposited thereon. This unit was integral in detecting the association of analytes, such as heparin, with the sensing molecules immobilized on the surface of substrate 14.

FIG. 5 illustrates schematically the arrangement of a detection system for 15 detecting analytes associated with the sensing molecules. Detection of the analyte was accomplished using an SPR system in standard Kretschmann geometry. A light source 26 capable of producing coherent, polarized light, such as a HeNe polarized laser U-1307P by Uniphase, was used to emit light. Light from coherent and polarized light source 26 was passed through two cylindrical lenses, 20 L1 and L2 respectively. This transformed the point image from the light source 26 into a line image. A third lens L3 was used to converge the line light beam from lenses L1 and L2 onto the surface of SPR unit 24, which included a thin silver film. The beam was TM polarized with respect to the plane of incidence. The reflected beam was converged by a fourth lens (L4) to form a parallel line beam that was directed through a cross polarizer 28 to a photodiode array (PDA) 25 system 30. A working embodiment of the invention used a Hamamatsu C-5964 photodiode array having 1024 silicon photodiodes, each 500 μm long and 25 μm wide. The cross polarizer 28 controlled the intensity of the incident light. This arrangement made it possible to scan the intensity of the reflected light with 30 respect to the angle of incidence without having to perform any mechanical manipulation while making measurements.

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The maximum scanning range for the illustrated system was  $\pm 5^\circ$  and was determined by the characteristics of the lenses L1, L2, and L3. This range can be changed by using different cylindrical lenses. The resolution and precision of the measurement system was determined by the above scanning range and the density of the diode array of the detector.

- Using the described configuration the resolution was about  $\pm 0.01^\circ$ . Solely for convenience, moderately sized system components were used: equilateral prism 40X40 mm<sup>2</sup>; the focal lengths of the lenses L1, L2, L3, and L4 were 6.35, 150, 60 and 60 mm, respectively. The total size of the system is around 1000 X 250 mm<sup>2</sup> but it can be reduced substantially using microoptics components including fiberoptic elements. PDA 30 was equipped with a control circuit which was powered and controlled by an external function generator and a pulse sequence from a National Instruments' DAQ-1200 card inside a IBM PC based computer. The data acquisition software was written using LabVIEW 4.0.
- 15 The output video signal from PDA 20 was synchronously digitized by the DAQ card and stored inside the computer.

- The SPR system illustrated in FIGS. 3-5 was designed to avoid using moving mechanical parts during the process of the measurement. The required precision limits the scanning angular range to about  $10^\circ$ . On the other hand, the angle of the attenuated total reflection (ATR) for the multilayer SPR unit 24 is  $45^\circ$  and  $65^\circ$  in the case of air and liquid coverage above the polymer film, respectively. Therefore the system is designed for detecting either in dry or wet flow cell conditions. The current results were performed at around  $45^\circ$  incidence, which allowed recording data only when the multilayer system was dry.

## V. EXAMPLES

- To further illustrate and describe the present invention, the following examples are provided. These examples are intended to be exemplary only, and 30 should not be construed to limit the invention to the particular aspects described therein.

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Example 1

This example illustrates the functionalization of graphite to establish that functionalization of such materials is possible using the methodology discussed above. A piece of pyrolytic graphite was freshly cleaved using transparent adhesive tape and coated with a solution of 0.5 % w/w N-hydroxysuccinimidyl 4-azidotetrafluorobenzoate (NHS-PFPA) in dry nitromethane by spinning at a speed of 1000 rpm. The coated graphite was baked at 60°C for 20 minutes and irradiated for 5 minutes using 254-nm lamps at ambient temperature under air. The graphite was then incubated in a 50- $\mu$ M solution of horseradish peroxidase (HRP) in NaHCO<sub>3</sub> buffer (pH 8.2) at 25°C for 3 hours and rinsed thoroughly with phosphate buffer (pH 7.0).

The enzymatic activity of the functionalized graphite was determined spectrophotically at 420 nm and 25°C in phosphate buffer using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) and hydrogen peroxide (1.8 mM ABTS/0.8mM H<sub>2</sub>O<sub>2</sub>). Assuming that the immobilized HRP had the same activity as the native HRP, the extent of immobilization of HRP was 2.1 ng/mm<sup>2</sup>.

A control experiment was performed as follows: A piece of freshly cleaved graphite was similarly baked, irradiated, and incubated with HRP solution. The enzyme-activity of the control was determined to be 0.4 ng HRP/mm<sup>2</sup>. Thus, the control was not treated with NHS-PFPA.

Samples and controls were examined using atomic-force microscopy (AFM). The atomic-force microscope was operated in air at ambient temperature. A representative AFM image of the sample is shown in FIG. 1A and of the control in FIG. 1B. In FIG. 1A, bright spheres correspond to immobilized HRP molecules. In FIG. 1B, only a few faint spheres were seen, indicating much less immobilization of the HRP molecules to the control surface.

Therefore, the NHS-PFPA allows substantial covalent attachment of HRP to the graphite surface.

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Example 2

This example illustrates that preselected patterns of functional groups can be immobilized on substrates. In this example, micron-sized patterns were "drawn" on a P3OT film containing NHS active ester using an electron beam.

- 5 A solution of 25.7 mg of P3OT and 1.8 mg of NHS ester (7 % w/w) in 0.6 mL of xylene was spin-coated on a silicon disc and dried at 60°C for 30 minutes. The resulting film was exposed to an electron beam to "draw" micron-sized patterns on the film (line width 0.5  $\mu\text{m}$ ; beam intensity 20  $\mu\text{C}/\text{cm}^2$ ). The film was then "developed" by dipping in xylene for 10 seconds, rinsing in  
10 isopropyl alcohol for 10 seconds and drying under a stream of nitrogen gas. The film was then immersed in a solution of 1.5 mg of amino-fluorescein and 6 mg of Et<sub>3</sub>N in 1 mL of EtOH for 4 hours. The film was then washed with EtOH, immersed in EtOH for 1 hour, washed again with EtOH, then air-dried.

- The sample film was observed and photographed using a fluorescence  
15 microscope equipped with a rhodamine filter set (excitation wavelength 510-560 nm, emission wavelength > 590 nm), yielding the results shown in FIG. 2A. The same sample film was observed and photographed using the fluorescence microscope equipped with a fluorescein filter set (excitation wavelength 450-490 nm, emission wavelength 515-565 nm) yielding the results shown in FIG. 2B.  
20 As can be seen, substantially identical patterns were observed having strong fluorescence at both the rhodamine excitation wavelength (FIG. 2A) and the fluorescein excitation wavelength (FIG. 2B).

- P3OT alone is strongly fluorescent at the rhodamine excitation wavelength but only weakly fluorescent at the fluorescein excitation wavelength. (This is  
25 why the films in this example were observed using a rhodamine filter set and a fluorescein filter set; strong fluorescence observed at the fluorescein excitation wavelength would necessarily be due to the presence of other molecules than just P3OT.) FIGS. 2A and 2B indicated that fluorescein became attached to the regions exposed to the electron beam.

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Example 3

This example is a control for example 2. A P3OT film was exposed to an electron beam (intensity 30  $\mu\text{C}/\text{cm}^2$ , line width 0.5  $\mu\text{m}$ ), developed, then treated with amino-fluorescein as described in example 2. The micron-sized patterns

5 "drawn" on the control P3OT film were identical to the patterns in Example 2.

When the control film was examined using a fluorescence microscope, strong fluorescence was observed at the rhodamine excitation wavelength (FIG. 2C), but only weak fluorescence was observed at the fluorescein excitation wavelength (FIG. 2D).

10 The results indicate that substantially no fluorescein became attached to P3OT in the absence of activated ester groups. Therefore, the presence of NHS active ester was required to obtain any substantial covalent coupling of the fluorescein to P3OT.

Example 4

15 This example describes the deposition of silver films on glass substrates, spincoating the metal substrates with polymeric material, and thereafter functionalizing the polymeric film by immobilizing protamine thereon. Thin silver films (56 nm thick) were deposited onto circular glass slides using a thermal evaporator. A solution of polystyrene (PS) in toluene (1 mg/mL) was

20 spin-coated on top of the silver film at 1000 rpm for 2 minutes. The film was baked at 90°C for 30 minutes. After cooling, a solution of 0.5% NHS-PFPA

ester in nitromethane was spin-coated on top of the PS film at 1000 rpm for 1 minute. The film was photolyzed in a Rayonet photoreactor at 254 nm for 5 minutes, rinsed with nitromethane for 10 seconds and blow-dried with N<sub>2</sub>. The

25 film was then immersed in a solution of protamine sulfate (Sigma) in pH 9.0 NaHCO<sub>3</sub> buffer (4 mg/mL) at 25°C for 1.5 hour, rinsed with water and N<sub>2</sub>-dried. This attached protamine on the silver substrate.

Example 5

This example describes the deposition of silver or gold films on glass substrates, spincoating the metal substrates with polymeric material, and thereafter functionalizing the polymeric film by immobilizing poly-L-lysine on the metal. Thin gold or silver films were deposited onto circular glass slides using a thermal evaporator. A solution of polystyrene in toluene (1 mg/mL) was spin-coated on top of the silver film at 1000 rpm for 2 minutes. The film was baked at 90°C for 30 minutes. After cooling, a solution of 0.5% NHS-PFPA ester in nitromethane was spin coated on top of the PS film at 1000 rpm for 1 minute. The film was photolyzed in a Rayonet photoreactor at 254 nm for 5 minutes, rinsed with nitromethane for 10 seconds and blow-dried with N<sub>2</sub>. The film was then immersed in a solution of poly-L-lysine in pH 9.0 NaHCO<sub>3</sub> buffer, rinsed with water and N<sub>2</sub>-dried. This attached poly-L-lysine to the metal films.

Example 6

This example describes the deposition of silver or gold films on glass substrates, spincoating the metal substrates with polymeric material, and thereafter functionalizing the polymeric film by immobilizing heparinase on the metal. Gold or silver thin films were deposited onto circular glass slides using a thermal evaporator. A solution of polystyrene in toluene (1 mg/mL) was spin-coated on top of the gold or silver film at 1000 rpm for 2 minutes. The film was baked at 90°C for 30 minutes. After cooling, a solution of 0.5% NHS-PFPA ester in nitromethane was spin-coated on top of the PS film at 1000 rpm for 1 minute. The film was photolyzed in a Rayonet photoreactor at 254 nm for 5 minutes, rinsed with nitromethane for 10 seconds and blow-dried with N<sub>2</sub>. The film was then immersed in a solution of heparinase in pH 8.3 NaHCO<sub>3</sub> buffer, rinsed with water and N<sub>2</sub>-dried. This attached heparinase on the silver substrate.

Example 7

This example describes an alternative method for functionalizing metals with analyte sensing molecules. Thin gold thin films (47nm thick) were deposited onto circular glass slides using a thermal evaporator. The film was reacted with 1-decanethiol to form a gold substrate having a pendent C-10 carbon

-30-

chain. A solution of NHS-PFPA ester (1) was then spin-coated onto the film followed by photolysis at 254 nm for 5 minutes. This resulted in the formation of a nitrene from the azide group of the NHS-PFPA ester, which underwent an insertion reaction with the pendent methyl group of 1-decanethiol. This attached 5 the NHS-PFPA ester to the gold substrate. The film was then immersed in a solution of protamine or poly-L-lysine or heparinase. This attached protamine or poly-L-lysine or heparinase to the gold substrate.

**Example 8**

This example describes the formation of pre-selected patterns of materials 10 on the surface of gold or silver substrates. A substrate made by evaporating gold or silver onto glass was reacted with 1-decanethiol to form a gold or silver substrate having a pendent C-10 carbon chain. A solution of NHS-PFPA ester was then spin-coated onto the film followed by photolysis at 254 nm using a UV mask aligner. This attached NHS-PFPA ester to the gold or silver surface in 15 preselected patterns. The film was then immersed into a solution of protamine or poly-L-lysine or heparinase. This attached protamine or poly-L-lysine or heparinase to the gold or silver substrate in pre-selected patterns.

**Example 9**

This example describes the formation of arrays on the surface of gold or 20 silver substrates. A substrate made by evaporating thin films of gold or silver onto glass was reacted with 1-decanethiol to form a gold or silver substrate having a pendent C-10 carbon chain. A solution of NHS-PFPA ester was then spin-coated onto the film followed by photolysis at 254 nm for 5 minutes. This attached NHS-PFPA ester to the gold or silver surface. The films were then 25 spotted with solutions of different sensing reagents, for example, protamine, heparinase and/ or glucose oxidase using a pipette or a mechanical spotting device. This generated arrays of sensing reagents on the gold or silver substrate.

**Example 10**

This example describes the immobilization of quaternary amines on 30 substrates for detecting heparin. Thin gold or silver thin films were spin-coated with a solution of polystyrene in toluene (1 mg/mL) followed by spin-coating a

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solution of compound 2. The film was photolyzed at 254 nm and rinsed with nitromethane. The film was then reacted with a solution of iodomethane in ether. This generated quaternary ammonium ions on the gold or silver surface.

**Example 11**

5 A substrate made from gold or silver deposited on glass was reacted with HS(CH<sub>2</sub>)<sub>x</sub>NMe<sub>2</sub> followed by reaction with iodomethane. This generated quaternary ammonium ions on the gold or silver surface.

**Example 12**

10 A substrate made from silver on glass was reacted with HS(CH<sub>2</sub>)<sub>x</sub>NMe<sub>2</sub> followed by reaction with iodomethane. This generated quaternary ammonium ions on the silver surface.

**Example 13**

15 A substrate made from gold or silver on glass was immersed in a solution of 11-mercaptoundecanoic acid (HS(CH<sub>2</sub>)<sub>11</sub>COOH) in ethanol for 24 hours followed by rinsing with ethanol and water. The film was then treated with a solution of protamine or poly-L-lysine or trioctylmethylammonium chloride in pH 8.2 NaCHO<sub>3</sub> buffer. This attached protamine or poly-L-lysine or trioctylmethylammonium chloride to the gold or silver surface.

**Example 14**

20 A solution comprising a polymer, such as polyvinylphenol, a bis-PFPA crosslinker and a sensing reagent, such as protamine or poly-L-lysine or trioctylmethylammonium chloride, was spin-coated onto gold or silver surfaces and then photolyzed. This generated a crosslinked polymer film with the sensing reagent embedded in the film.

25 **Example 15**

BK-7 glass substrates with a flatness of 1 wavelength were obtained from Argus International. A thin silver film (55 to 60 nm) was directly deposited on the glass surface by vacuum thermal deposition at pressures around 5-7 Torr. The substrate temperature during evaporation was around 300 K. The silver coated samples were further processed to bind protamine sulfate to its surface as described above in example 4. Samples were mounted in a sample holder

designed so that the silver coating with the bound protamine formed one wall of the flow cell of FIG. 3. The Teflon-flow cell was sealed using a Viton-1 compression O-ring fitting. Optical contact with the BK-7 glass prism was accomplished using index matching fluid (RESOLVE by Stephens Scientific).

- 5 All of the system was mounted onto a rotational optical stage capable of manually adjusting the rotation angle with a 0.5° precision.

Detection of the analyte was accomplished using a surface plasmon resonance (SPR) system in standard Kretschmann geometry as described above. Various materials, including sample containing analyte, water, and N<sub>2</sub> were fed 10 to the flow-through cell 12 using a 6-to-1 electromechanical valve system that was computer controlled.

- 15 After mounting the sample onto the flow cell the optical system was adjusted to maximize the sensitivity with the set of lenses available. A valve control time sequence was then controlled by the computer program so that at certain times a required liquid or gas was flowed through the cell.

- 20 A series of tests were run using the flow through sample and detection system discussed above. The results of these experiments are presented in Table 1 below. Table 1 has the following entries: "N<sub>2</sub>" refers to flowing ultrahigh pure nitrogen gas through the flow through cell; "water" refers to deionized H<sub>2</sub>O (18 MΩ); "tris" refers to a tris-sulfate buffer solution; "heparin" refers to a solution comprising tris buffer and heparin. The numbers in parentheses refer to valves that were opened to introduce each material into the flow-through cell.

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TABLE 1

STEP	VALVE (#)	TIME, MIN.
1.	closed (6)	10
2.	N <sub>2</sub> (0)	5
3.	closed (6)	5
4.	N <sub>2</sub> (0)	5
5.	closed (6)	5
6.	water (2)	5
7.	N <sub>2</sub>	30
10	closed (6)	10
8.	water (2)	5
9.	N <sub>2</sub> (0)	30
10.	closed (6)	10
11.	tris (1)	5
15	water (2)	10
12.	N <sub>2</sub> (0)	30
13.	closed (6)	10
14.	tris (1)	5
15.	water (2)	10
16.	N <sub>2</sub> (0)	30
20	closed (6)	10
17.	heparin (3)	5
18.	water (2)	10
20.	N <sub>2</sub> (0)	30
19.	closed (6)	10
22.	heparin (3)	5
25	closed (6)	5
23.	water (2)	10
24.	N <sub>2</sub> (0)	30
25.	closed (6)	10
26.	heparin (3)	5
30	closed (6)	15
27.	water (2)	10
28.	N <sub>2</sub> (0)	30
29.	closed (6)	10
30.	heparin (3)	5
31.	closed (6)	15
32.	water (2)	10
35	N <sub>2</sub> (0)	30
33.	closed (6)	10

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A series of control runs, as well as flowing heparin solutions through the flow-through cell, were then carried out. The flow through cell first was analyzed with all valves closed. Thereafter, the flow through cell was flushed with N<sub>2</sub> to determine the temperature dependence of the resonance condition.

- 5      The temperature dependence of the optical dielectric constant of the metal layer, as well as the coatings above the silver thin film, must be determined so that the size of the effect can be distinguished from when analytes bind to the surface.

After the flow-through cell was flushed with N<sub>2</sub>, the cell was then flushed with deionized water and thereafter dried to see what changes occurred in the  
10     SPR position due to residual humidity. This flushing and drying sequence also was done to understand the amount of time that might be required to analyze a sample.

- The last preliminary set of control cycles was done using tris-sulfate buffer solution containing no heparin. The sensor surface first was exposed to  
15     tris-buffer solution. Thereafter, the flow-through cell was flushed with deionized water and dried using a flow of UHP N<sub>2</sub> gas. This sequence was tested to investigate the influence of running a highly ionic solution on the multilayer structure. This sequence also served as a main control indicator for the test runs with heparin/tris-buffer solution. Flushing with water was used both in the  
20     control runs and in the actual test runs to remove any residual salts which might condense on the sensor surface during the drying process.

FIG. 6 illustrates the data recorded by the photodiode array. On the horizontal axis the diode number is displayed which corresponds to a certain angle of incidence of the laser light. The recorded voltage is reported on the vertical axis, which corresponds to the light intensity at the specified diode. The curve minima at about diode number 500 corresponds to the surface plasmon resonance condition. The movement of the dip in intensity at which SPR occurs will be of main interest.

FIG. 7 illustrates the peak position dependence on time as the flow cell is subjected to different conditions. At the time when the sensor is under liquid the SPR position is out of the range of incidence angles scanned when the surface is

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under liquid. This is represented by the measurement being off scale (position reading showing 400). The position of the SPR minima stays the same after flowing controls through the cell, namely water and tris-buffer (at around  $492 \pm 2$ ), and shifts to a higher number when the sensor surface is exposed to heparin.

5       The curve minima dependance on the heparin exposure time is illustrated in FIG. 8. The SPR shift saturates for times around 30 minutes. This FIG. 8 further illustrates that the SPR detection system can be used for determining concentrations of analytes, in this case heparin, by using a calibrated curve.

## VI. APPLICATIONS

10       Sensors made according to the present invention are useful for detecting heparin and heparin concentrations. However, such sensors also can have plural sensing mechanisms on one single unit. With respect to a sensing unit that has plural sensing mechanisms, the heparin sensor as described herein could be combined with other sensing mechanisms, such as a glucose sensor, so that  
15       heparin and glucose blood levels can be monitored.

The present sensors are small, and therefore can be produced in a handheld unit, or other transportable device. Alternatively, the sensing unit can be produced to be integrated with diagnostic equipment already available. For example, a sensing chip can be produced that is integrated with other electronic  
20       portions of existing equipment.

Moreover, the sensors described herein can be used both in invasive and non-invasive applications, that is external to the human body, or *in vivo*. With respect to non-invasive methods, blood samples can be taken from the patient and the heparin blood levels detected.

25       In a non-fully invasive application, the heparin sensor could be attached to a catheter used to divert blood from a patient's circulatory system through the sensor. This allows blood to be continuously circulated through the catheter, thereby through the heparin sensor, to continuously monitor heparin levels in line. This is possible either by providing a sensor having sufficient heparin  
30       sensing units to monitor heparin levels for a given period of time. Alternatively, the heparin sensors of the present invention are reversible, i.e., they can first

detect heparin levels, followed by a regeneration process whereby the heparin sensors are fully regenerated for subsequent use. Regeneration generally is accomplished by flushing the sensor with a salt solution, particularly NaCl. This disrupts the ionic interaction between, for example, protamine and heparin, 5 allowing the heparin to be back-extracted. This regenerates free protamine molecules for subsequent detection of heparin.

The sensors also can be coupled to a feedback system. For example, if the sensor is solely designed for monitoring heparin levels, a heparin pump can be coupled to the circulatory system of a patient. As heparin levels drop, the pump 10 is activated to increase the heparin concentration in the blood. Similarly, the heparin sensor could be a dual or plural sensing unit with a feedback system. In one embodiment, a heparin sensor that also includes a glucose sensing is coupled with a feedback system for introducing both heparin and insulin into the patient's blood stream.

15 While the invention has been described in connection with preferred embodiments and multiple examples, it will be understood that it is not limited to those embodiments. On the contrary, it is intended to cover all alternatives, modifications, and equivalents as may be included within the spirit and scope of the invention as defined by the appended claims.

## WE CLAIM:

1. A sensor, comprising:  
an analyte detector comprising sensing materials immobilized thereon for ionically or covalently binding heparin; and  
5 means for detecting heparin after it binds to the sensing materials.
2. The sensor according to claim 1 wherein the analyte further comprises sensing materials for sensing analytes other than heparin.
3. The sensor according to claim 2 wherein the analyte detector includes sensing material for sensing glucose.
- 10 4. The sensor according to claim 3 wherein the sensing material for sensing glucose comprises glucose oxidase.
5. The sensor according to claim 1 wherein the sensing materials are selected from the group consisting of protamine, heparinase, poly-L-lysine, and long-chain quaternary ammonium salts.
- 15 6. The sensor according to claim 1 further including means for flushing the sensor after the sensing molecules have ionically bound to heparin.
7. The sensor according to claim 6 wherein the means for flushing the sensor comprise means for flushing the sensor with salt solutions.
8. The sensor according to claim 6 wherein the sensing molecules are 20 protamine.
9. The sensor according to claim 1 wherein the means for detecting heparin is surface plasmon resonance.
10. The sensor according to claim 1 further comprising a catheter for flowing blood from a patient to the analyte detector.
- 25 11. The sensor according to claim 10 wherein the catheter continuously flows blood from a patient to the analyte detector.
12. The sensor according to claim 1 and further comprising an analyte pump for administering analyte to a patient when analyte levels detected by the sensor are lower than a predetermined threshold.
- 30 13. A sensor, comprising:

an analyte detector comprising sensing materials immobilized thereon for ionically or covalently binding heparin, the sensing materials being selected from the group consisting of protamine, heparinase, polylysine, poplybrene, quaternary ammonium salts, and mixtures thereof; and

5 surface plasmon resonance means for detecting heparin after it binds to the sensing materials.

14. The sensor according to claim 13 wherein the analyte further comprises sensing materials for sensing analytes other than heparin.

15. The sensor according to claim 14 wherein the analyte detector includes 10 sensing material for sensing glucose.

16. The sensor according to claim 15 wherein the sensing material for sensing glucose comprises glucose oxidase.

17. The sensor according to claim 13 further including means for reversing the interaction of heparin with the sensing molecules.

15 18. The sensor according to claim 13 further comprising at least one catheter fluidly coupled to the sensor and to a patient for flowing blood from the patient to the analyte detector.

19. The sensor according to claim 13 and further comprising an analyte pump for administering analyte to a patient when analyte levels detected by the 20 sensor are lower than a predetermined threshold.

20. A handheld sensor, comprising:  
an analyte detector;  
heparin sensing materials immobilized on the analyte detector for ionically or covalently binding heparin, the sensing materials being selected from the 25 group consisting of protamine, heparinase, polylysine, poplybrene, quaternary ammonium salts, and mixtures thereof;

analyte sensing materials other than heparin immobilized on the analyte detector; and

means for detecting heparin and analytes other than heparin after binding to 30 the sensing materials.

21. The sensor according to claim 20 wherein the analyte detector includes sensing materials for sensing glucose.
22. The sensor according to claim 21 wherein the sensing material for sensing glucose comprises glucose oxidase.
- 5 23. The sensor according to claim 20 further including means for reversing the interaction of heparin with the sensing molecules.
24. A method for detecting heparin, comprising:
  - providing a substrate capable of undergoing an insertion reaction with a nitrene;
- 10 coating the substrate with an activated perfluorophenyl azide;
  - exposing the substrate and perfluorophenyl azide to a reaction energy source, thereby forming a functionalized substrate;
  - reacting the functionalized substrate with heparin sensing molecules, thereby forming a heparin sensitive substrate;
- 15 coupling a detector to the heparin sensitive substrate to form a sensor;
  - exposing the sensor to samples containing heparin so that the sensing molecules interact with the heparin; and
  - detecting interactions between heparin and the sensing molecules.
- 20 25. The method according to claim 24 wherein the perfluorophenyl azide is an NHS-PFPA.
26. The method according to claim 24 wherein the reaction energy source is selected from the group consisting of light and an electron beam.
27. The method according to claim 24 wherein the heparin sensing molecules are selected from the group consisting of protamine, heparinase, polylysine, polybrene, quaternary ammonium salts, and mixtures thereof.
- 25 28. The method according to claim 24 wherein the detector is an SPR detector.

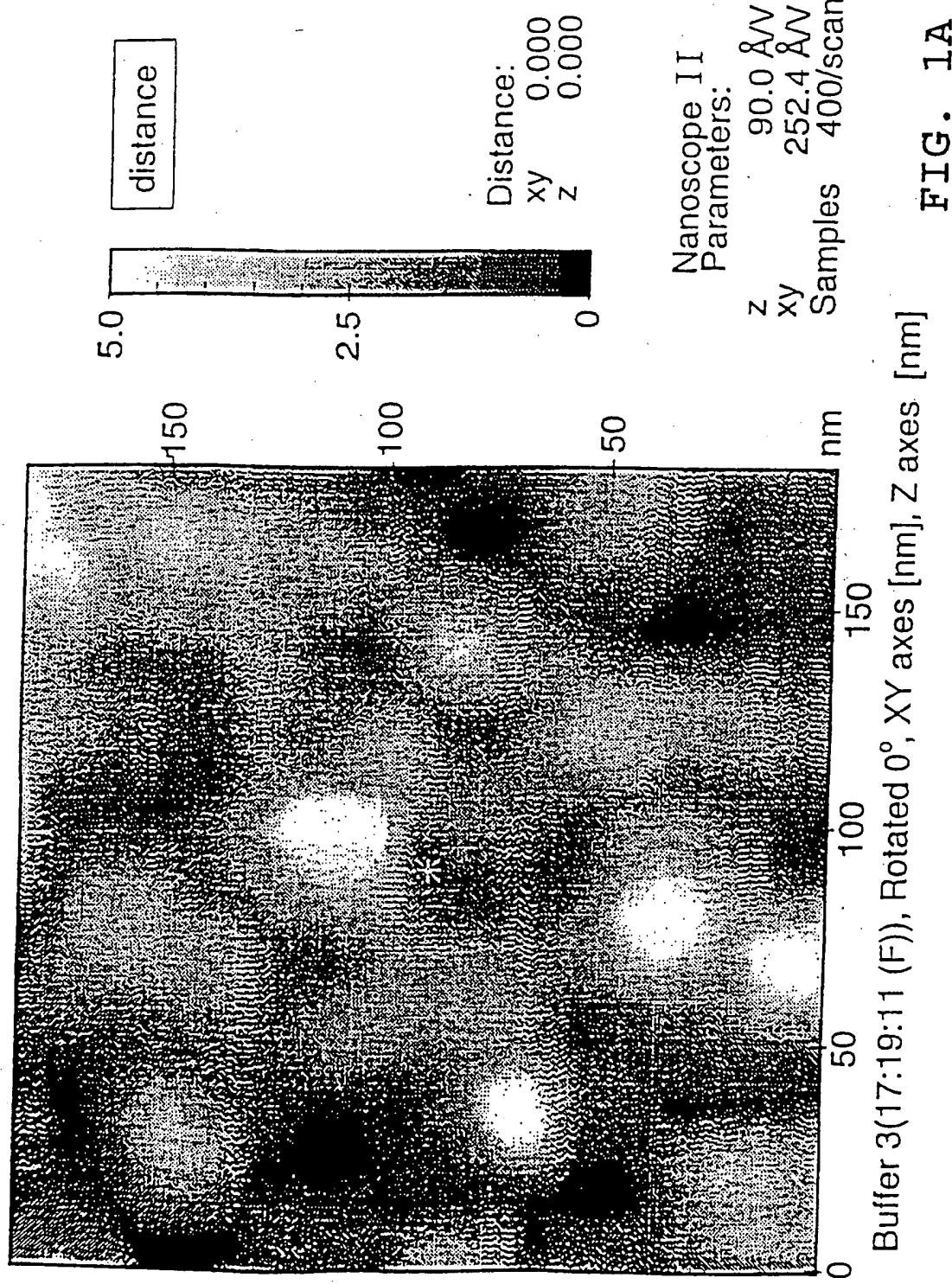
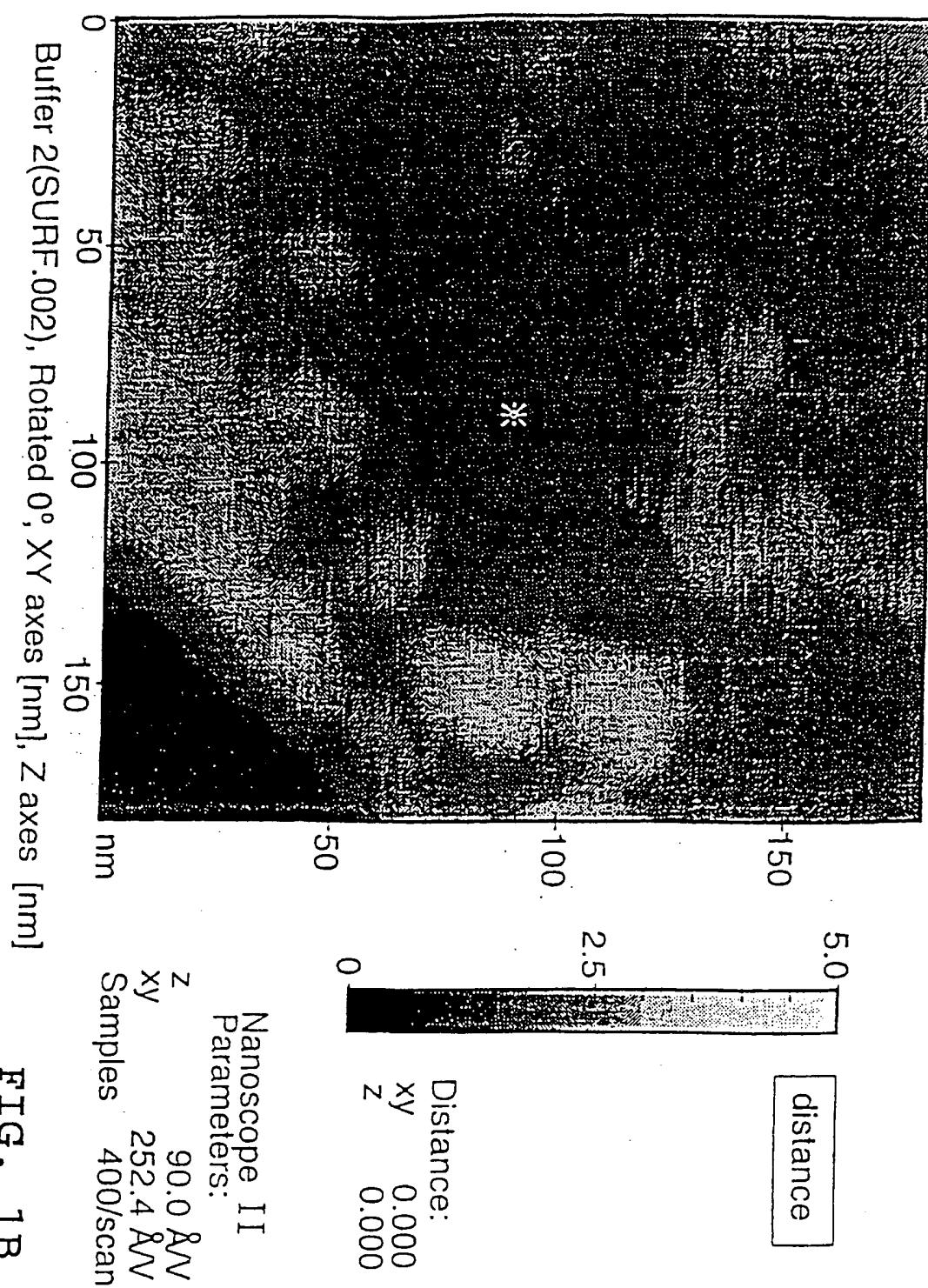


FIG. 1A

2/10



Buffer 2(SURF.002), Rotated 0°, XY axes [nm], Z axes [nm]

FIG. 1B

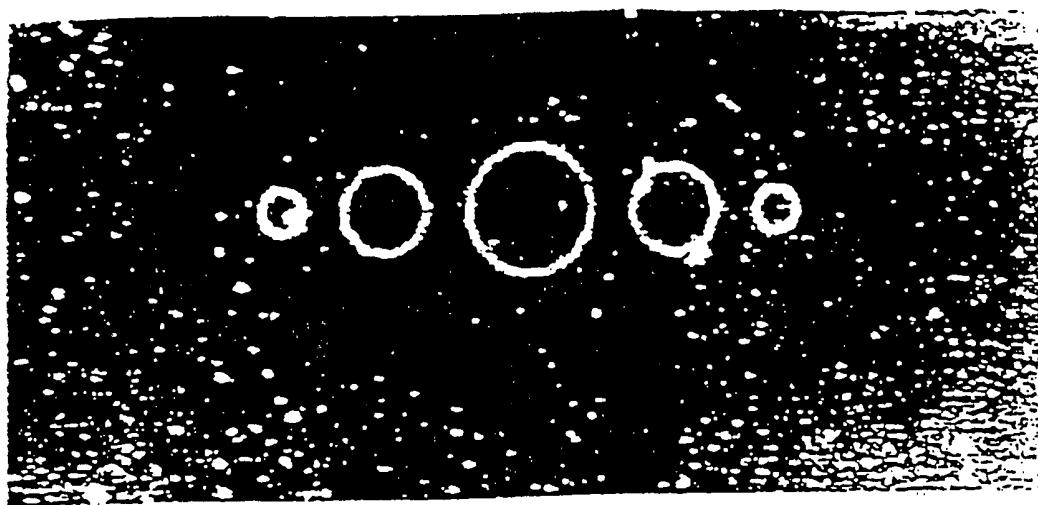


FIG. 2A

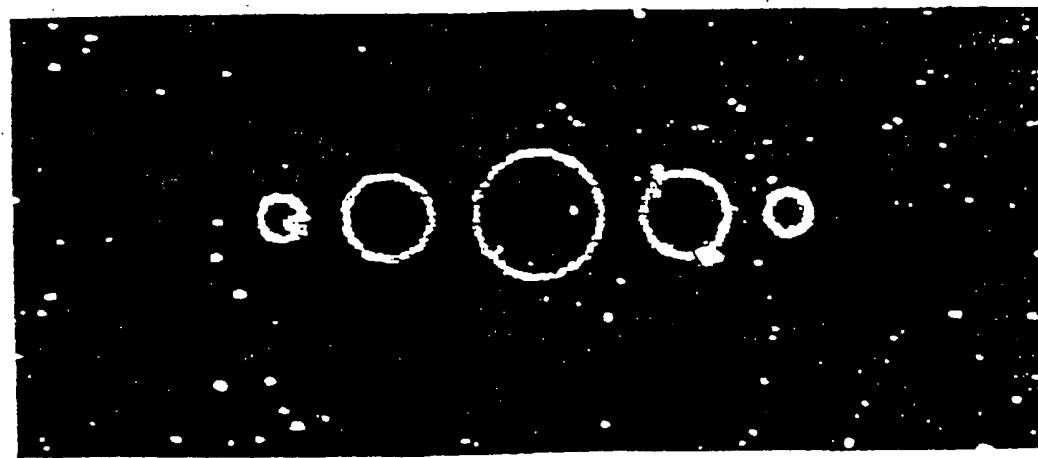


FIG. 2B

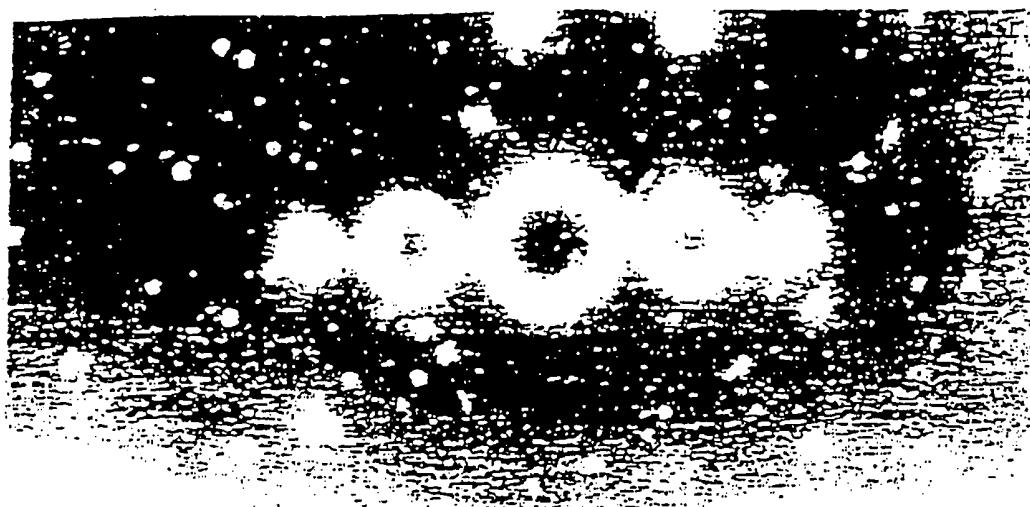


FIG. 2C

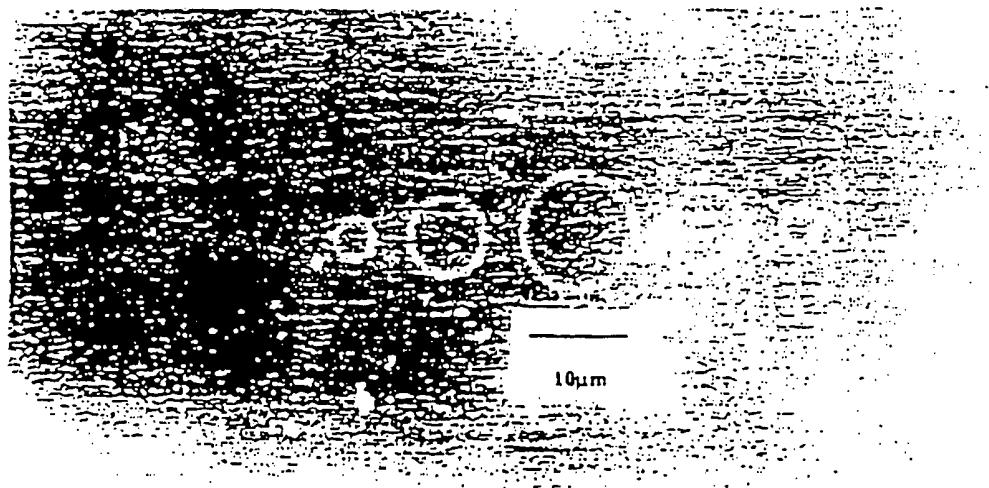


FIG. 2D

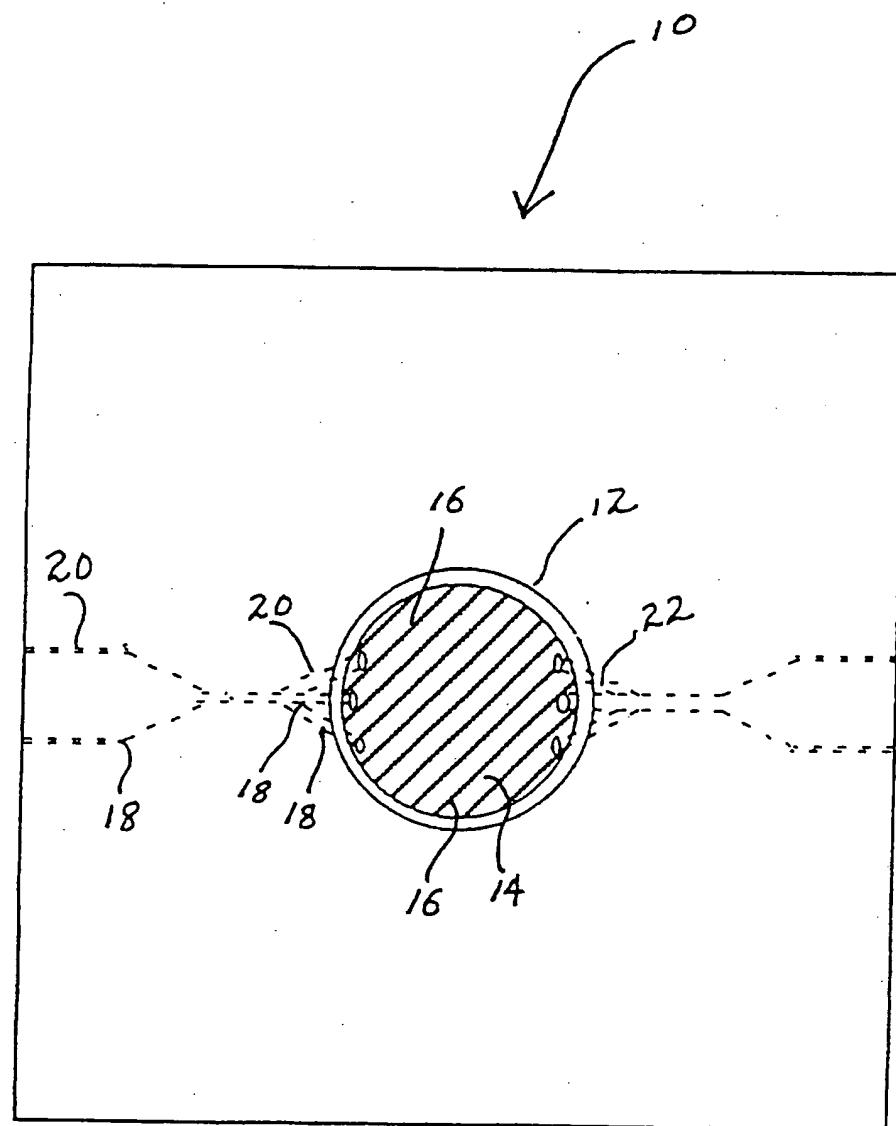


FIG. 3

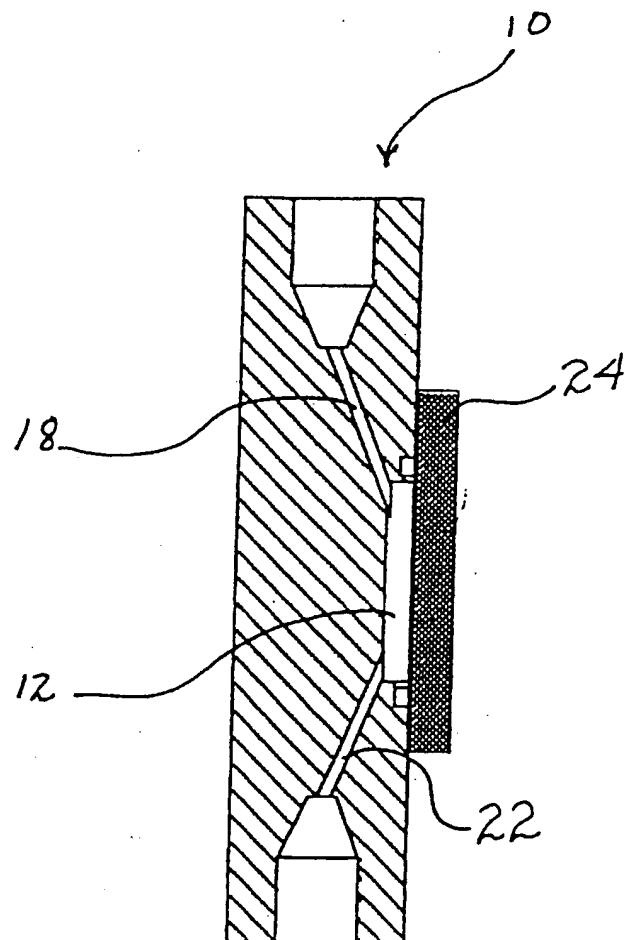


FIG. 4

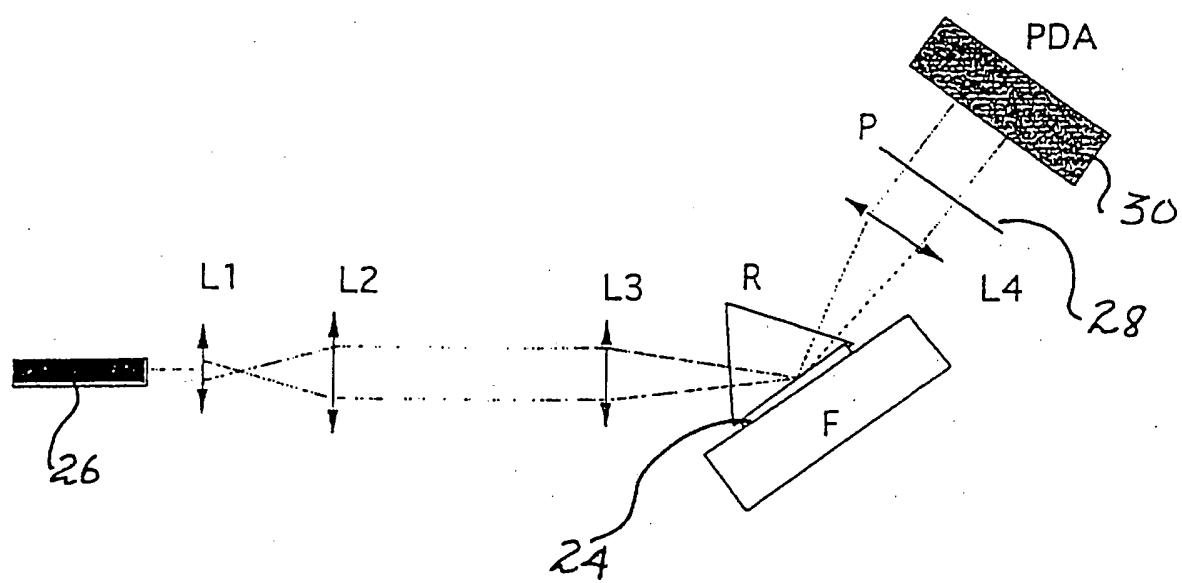


FIG. 5

2.000

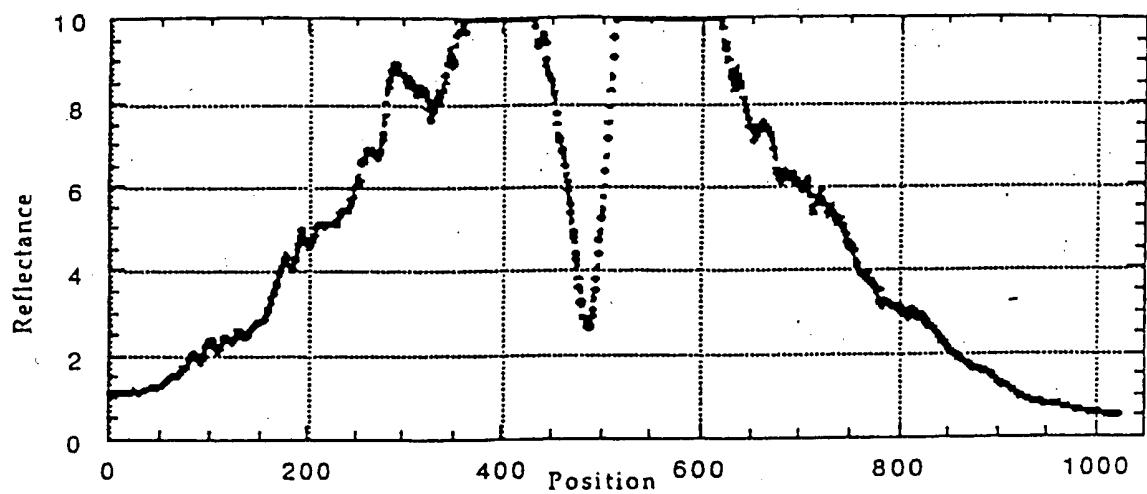


FIG. 6

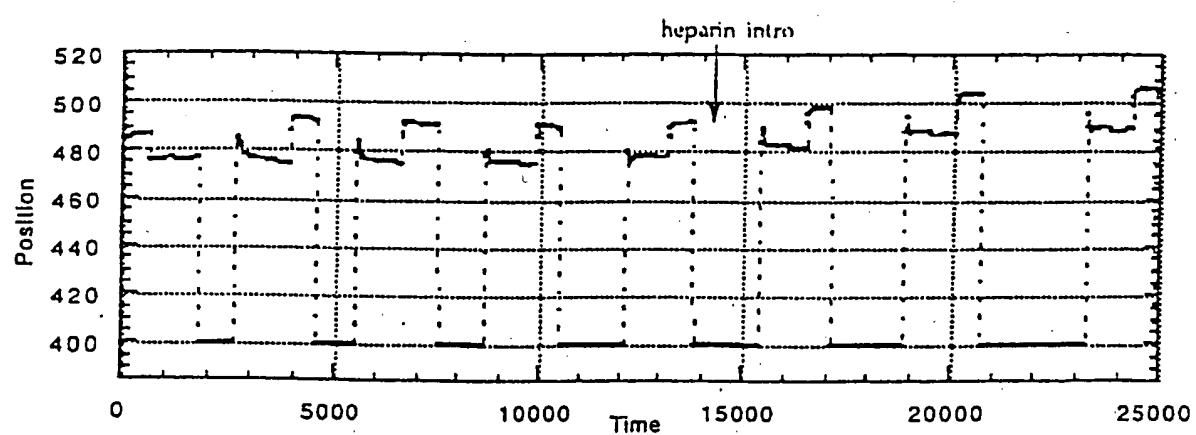
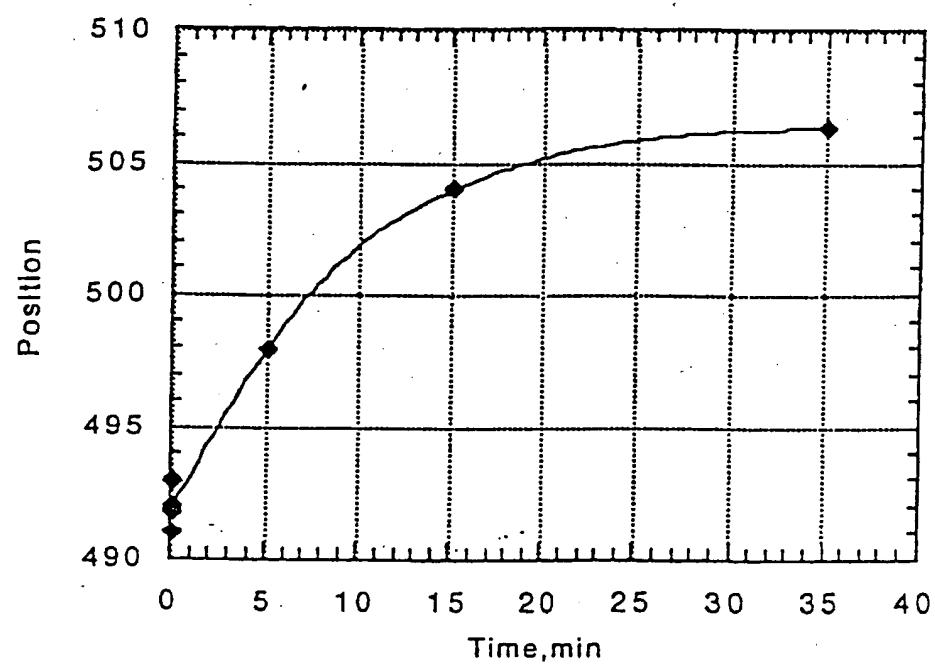


FIG. 7



**FIG. 8**

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/12982

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 21/17

US CL :Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/91, 93, 94, 95, 164, 166; 435/13, 14, 25, 287.1, 287.9, 288.7; 422/57, 82.05, 82.06, 82.09, 82.11

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database CAS ONLINE, Chemical Abstracts, (Columbus, Ohio, USA), AN 122:255063 CA, WANG ET AL, 'Optical detection of macromolecular heparin via selective coextraction into polymeric films,' abstract, Anal. Chem. 1995, 67(3), 522-7.	1, 5 -----
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Y	detection of macromolecular heparin via selective coextraction into polymeric films,' abstract, Anal. Chem. 1995, 67(3), 522-7.	2-4, 6-7, 10-12, 20-23
--		
X	Database CAS ONLINE, Chemical Abstracts, (Columbus, Ohio, USA), AN 122:255383 CA, VAN KERKHOF ET AL, 'The ISFET based heparin sensor with a monolayer of protamine as an affinity ligand,' abstract Biosens. Bioelectron., 1995, 10(3/4), 269-82.	1, 5, 8 -----
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Y	'The ISFET based heparin sensor with a monolayer of protamine as an affinity ligand,' abstract Biosens. Bioelectron., 1995, 10(3/4), 269-82.	6-7, 10-12
--		

 Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance		
*E* earlier document published on or after the international filing date	X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*O* document referring to an oral disclosure, use, exhibition or other means	A*	document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

05 SEPTEMBER 1997

Date of mailing of the international search report

17 OCT 1997

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**INTERNATIONAL SEARCH REPORT**

International application No.  
PCT/US97/12982

**A. CLASSIFICATION OF SUBJECT MATTER:**  
US CL :

436/91, 93, 94, 95, 164, 166; 435/13, 14, 25, 287.1, 287.9, 288.7; 422/57, 82.05, 82.06, 82.09, 82.11

**B. FIELDS SEARCHED**

Electronic data bases consulted (Name of data base and where practicable terms used):

**APS, CAS ONLINE**

search terms: heparin; polylysine or polybrene or heparinase or protamine or ammonium; glucose; oxidase; plasmon or spr; perfluorophenyl azide#

## **INTERNATIONAL SEARCH REPORT**

International application No.

PCT/US97/12982

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	Database CAS ONLINE, Chemical Abstracts, (Columbus, Ohio, USA) AN 124:134637 CA, YUN ET AL, 'Clinical application of disposable heparin sensors Blood heparin measurements during open heart surgery,' abstract, ASAIO J., 1995, 41(3), M661-M664.	1, 5 _____
X, P - Y, P	US, A, 5,629,213 (KORNGUTH ET AL) 13 May 1997 (13.05.97), see abstract.	1, 2, 5, 9, 13, 14 _____
Y	US, A, 5,298,022 (BERNARDI) 29 March 1994 (29.05.94), see abstract and column 4, line 9.	3-4, 10-12, 15-23 2-4, 10-12, 18-22
X - Y	US, A, 5,465,151 (WYBOURNE ET AL) 07 November 1995 (07.11.95), see brdgte columns 3-4; column 5, lines 1-5; column 15, lines 20-25; column 21, line 30; and column 22, lines 17-44.	1, 2, 5 _____
Y	US, A, 5,494,829 (SANDSTROM ET AL) 27 February 1996 (27.02.96), see column 11, lines 5-10; column 32, lines 35-36; and column 36, line 22.	3-4, 6-7, 9, 13-17, 20-28 2-4, 14-16, 20-22